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13. ABSTRACT *(Maximum 200 Words)*

The goals of this project are to prepare and test novel bifunctional chelates based on DOTA to antibodies directed to CEA (carcinoembryonic antigen) and Her2/neu. The conjugates will be radiolabeled with ^{111}In for tumor imaging and ^{90}Y for tumor therapy. In the first year of the project we have shown that the CEA positive MCF7 cell line transfected with Her2/neu can be grown in nude mice injected with estrogen pellets and used as a tumor model. Since we have previously conjugated anti-CEA antibody T84.66 with DOTA and shown it to target CEA positive tumors, we began our work with the anti-Her2/neu antibody 4D5. In the second year of the project, we have extended these studies to the humanized version of 4D5, Herceptin, and shown that good tumor targeting can be achieved even for parental MCF7 cells that don't over-express Her2/neu. We have also shown that ^{90}Y-anti-Her2/neu therapy is about equally effective to ^{90}Y-anti-CEA therapy.

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4.0 Introduction

Breast cancer can be targeted with radiolabeled anti-tumor antibodies. In this project we chose anti-CEA antibody T84.66 for targeting CEA positive breast cancers (about 50% are CEA positive) and anti-Her2/neu antibody 4D5 for Her2/neu positive breast cancer (about 30% are Her2/neu positive). Both antibodies are well characterized and have been used clinically, chimeric T84.66 and humanized 4D5 (Herceptin). The novel aspects of this project are the use of novel chelates to improve the biodistributions and tumor to blood ratios of the radiolabeled antibodies. The radioisotopes are ^{111}In (2.8 day half life, pure gamma emitter) for imaging and ^{90}Y (64h half life, pure beta emitter) for therapy. This is a preclinical study to determine the optimum chelate for each antibody. The original animal model was a CEA positive/Her2/neu positive cell line grown as a xenograft in nude mice. Since work over the first year suggested that this cell line was sensitive to cold 4D5 in vivo, we also explored use of the parent cell line MCF7, which is also CEA and Her2/neu positive. In addition, we began using the humanized version of 4D5, Herceptin.

5.0 Body

Cell lines. The MCF7/Her2/neu cell line was obtained from Dr. Dennis Slamon at UCLA. The cell line is positive for both CEA and Her2/neu which was transfected into the parent line MCF7 (1). These cells were analyzed for Her2/neu by FACS using the 4D5 antibody. The results (**Figure 1B**) show intense staining for Her2/neu compared to the parent cell line (**Figure 1D**).

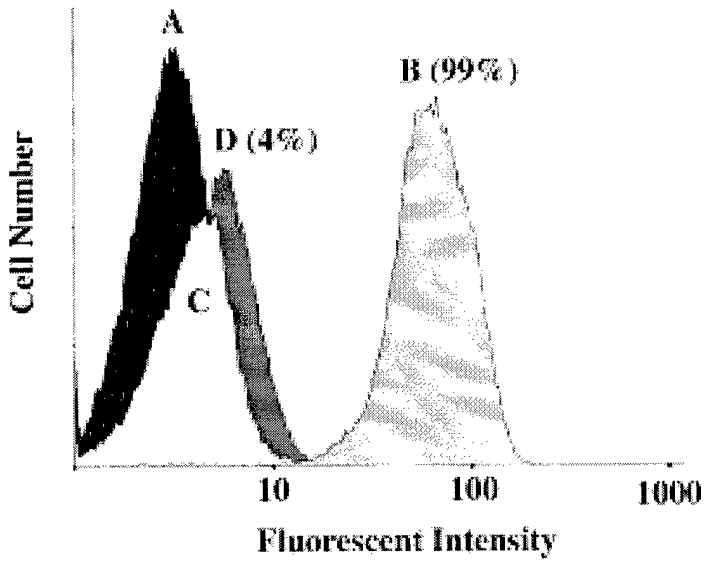

Figure 1. FACS analysis of MCF7/Her2/neu and MCF7 cells with anti-Her2/neu antibody 4D5. **A**. MCF7/Her2/neu cells, secondary antibody only. **B**. MCF7/Her2/neu cells stained with 4D5 (99% positive). **C**. Parental line MCF7, secondary antibody only. **D**. Parental line MCF7 stained with 4D5 (4 % positive).

Antibody conjugate. 4D5 or Herceptin was conjugated to DOTA (1,4,7,10-tetraazacyclododecane- N,N',N'',N'''tetraacetic acid) using our previously published active ester method (2). Briefly, the antibody (2 mg in 1 mL of PBS) was mixed with EDC (1-ethyl-3-[3-dimethylamino)propyl] carbodiimide) and sulfo-N-hydroxysuccinimide at a ratio of 1:100 for 1 h at room temperature and then dialyzed into 0.2 M ammonium acetate pH 5 buffer. The DOTA conjugated antibody was radiolabeled with either ^{111}In or ^{90}Y in the ammonium acetate buffer for 1 h at 43°C. The radiolabeled antibody was separated from free isotope after the addition of 10 mM DTPA (diethyltriaminopentaacetic acid) by gel filtration chromatography (TosoHaas TSK G2000, 10 um, 7.5 x 300 mm) in normal saline at a flow rate of 0.5 mL/min and monitored by A280 nm and radioactivity. Based on this analysis, incorporation of radioisotope was 80%. The number of chelates per antibody (5.0) was determined by using radiotracer tagged $^{111}InCl_3$. The immunoreactivity of the radiolabeled antibody was shown to be 95% based on a cell binding assay. Based on these analyses, we conclude that 4D5 or Herceptin can be conjugated to DOTA without loss of immunoreactivity and can be efficiently radiolabeled with either ^{111}In or ^{90}Y.

Animal biodistributions. Animal bidistributions for 4D5 using the MCF7/Her2/neu cells in nude mice have been published by us (4). New studies using the MCF7 parental line and Herceptin have just been completed. Groups of five mice per time point bearing MCF7 xenografts were injected with 5.0 uCi of ^{111}In-labeled DOTA-Herceptin (2.7 ug antibody per mouse) via tail vein. Animals were sacrificed at 0, 5, 24, 48, 72 and 96 hours post injection. Tumors were dissected and the major organs and blood weighed and the activity measured. The activity expressed in percentage injected dose per gram of tissue was then calculated. The mean values were used to construct biodistribution curves for tumors and normal organs. The results are shown in **Table 1** and **Figure 2**. Tumor uptake for the low Her2/neu expressing cell line (MCF7) is almost equivalent to the her2/neu over-expressing line, suggesting that both types of patients would qualify for treatment.

Table 1. Biosdistribution of In^{111} labeled DOTA-conjugated Herceptin in nude mice bearing MCF7 xenografts.

Organ	Time (h)			
	0 &	24	48	72
Blood	40.91 (3.76)	15.83 (2.80)	11.96 (2.43)	10.84 (3.01)
Liver	7.60 (0.48)	5.05 (0.58)	5.39 (1.54)	6.90 (1.45)
Spleen	5.59 (1.00)	3.76 (1.04)	3.31 (0.67)	3.15 (0.82)
Kidney	6.62 (0.78)	4.80 (0.73)	4.17 (0.70)	4.10 (0.29)
Lung	11.16 (2.09)	6.77 (2.31)	4.92 (1.15)	4.28 (0.89)
Bone	1.84 (0.48)	1.78 (0.34)	1.52 (0.52)	1.31 (0.21)
Tumor	0.95 (0.22)	16.91 (2.64)	25.4 (5.60)	24.94 (3.31)
Carcass	1.54 (0.27)	2.71 (0.44)	2.33 (0.38)	2.24 (0.36)

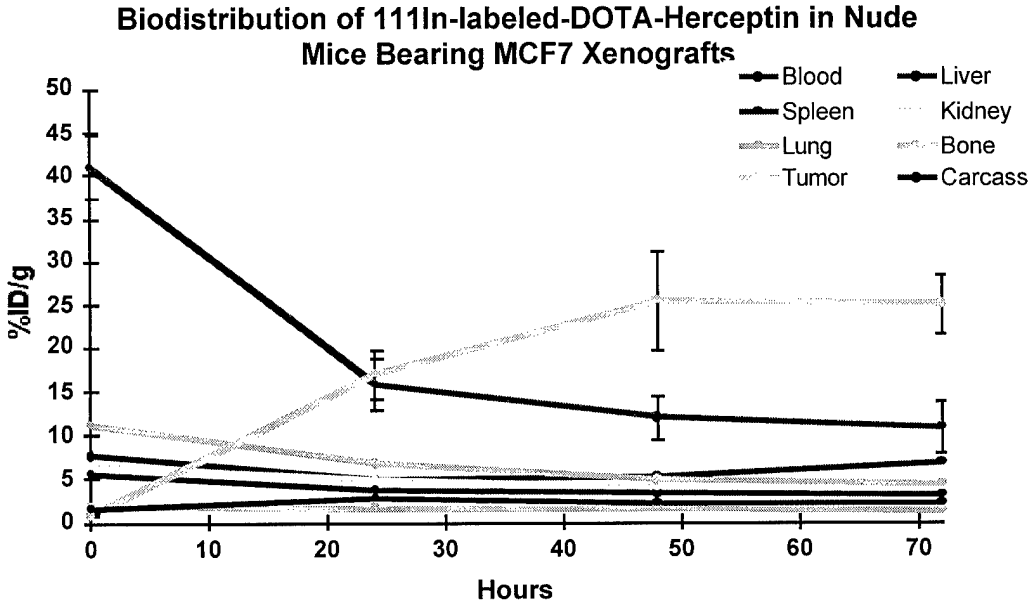

Figure 2. Biosdistribution of In^{111} labeled DOTA-conjugated Herceptin in nude mice bearing MCF7 xenografts.

Radioimmunotherapy in the animal model. Using the same model, 50 or 100 uCi of ^{90}Y labeled DOTA-4D5 or Herceptin was administered to groups of 9 animals. Control groups were injected with 100 uCi of ^{90}Y labeled DOTA-Leu16 (irrelevant antibody), unlabeled DOTA-4D5 (3 ug, the same amount of antibody as in the radiolabeled group) or saline. Tumor volumes (L x $W^2/2$)were measured twice weekly and relative tumor volumes calculated (compared to RIT at day 1. The results with ^{90}Y labeled DOTA-4D5 (4) show that mice injected with 50 uCi of ^{90}Y-labeled DOTA-4D5 had a two-fold reduction in tumor growth compared to control groups at the end of 37d. For those treated with 100 uCi of radiolabeled antibody, tumor growth was reduced 2.7 fold. Since this was only a single injection, tumor regrowth started to occur at 19d. There were no tumor cures with the single injection of ^{90}Y labeled DOTA-4D5. The results with ^{90}Y labeled DOTA-Herceptin are similar and are now being written up for publication.

6.0 Key Research accomplishments

- We have shown that radiolabeled anti-her2/neu antibodies, either 4D5 or Herceptin, can be used to target and treat her2/neu positive tumors in an animal model.
- We have shown that the targeting (animal biodistributions) are equivalent for either over-or "normal" expression levels of her2/neu in the tumor.
- We have shown that DOTA is an appropriate chelate for the targeting and therapy studies.

7.0 Reportable outcomes

a) manuscripts: Tsai, S.W., Sun, YY., Wlliams, L.E., Raubitschek, A.A., Wu, A.M., and Shively, J.E. (2000). Biodistribution and radioimmunotherapy of human breast cancer xenografts with radiometal-labeled DOTA conjugated anti-her2/neu antibody 4D5. *Bioconj. Chem.*, *11* 327-334.

b) patents: none.

c) degrees obtained that are supported by this award: none

d) development of cell lines, tissue or serum repositories: none

e) informatics such as databases and animal models: none

f) funding applied for based on work supported by this award: none

g) employment or research opportunities applied for and/or received on experiences/training support by this award: none

8.0 Conclusions.

The results confirm that the animal model is suitable for evaluating radioimmunotherapy of ^{90}Y labeled DOTA-4D5 or Herceptin. We are now in a position to compare these results to other chelates conjugated to 4D5 and to ^{90}Y labeled DOTA-T84.66 (anti-CEA antibody). We are writing an IND to begin imaging in Her2/neu positive patients and will expand these studies to therapy if the results merit the study.

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3. Williams, L. E., Primus, F. J., Wong, J. Y. C., Wu, A. M., Odon-Maryon, T. L., Johnson, D. K., Hefta, L. J. F., Shively, J. E., and Raubitschek, A. A. Biodistribution of an indium-111 or yttrium-90-labelled chimeric anti-carcinoembryonic antigen monoclonal antibody (cT84.66) following its large scale production in a bioreactor, Tumor Targeting. *2:* 116-124, 1996.

4. Tsai, S.W., Sun, YY., Wlliams, L.E., Raubitschek, A.A., Wu, A.M., and Shively, J.E. (2000). Biodistribution and radioimmunotherapy of human breast cancer xenografts with radiometal-labeled DOTA conjugated anti-her2/neu antibody 4D5. *Bioconj. Chem.*, *11* 327-334.

10.0 Appendices:

See attached manuscript.

Biodistribution and Radioimmunotherapy of Human Breast Cancer Xenografts with Radiometal-Labeled DOTA Conjugated Anti-HER2/neu Antibody 4D5

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Biodistribution and Radioimmunotherapy of Human Breast Cancer Xenografts with Radiometal-Labeled DOTA Conjugated Anti-HER2/neu Antibody 4D5

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HER2/neu oncogene encodes a 185 kDa trans-membrane protein which is overexpressed in 20−30% of breast and ovarian cancers and portends a poor prognosis. We have studied the targeting and therapy of this oncoprotein with 4D5, a murine monoclonal antibody which recognizes a distinct epitope on the extracelluar domain of HER2/neu. We conjugated the antibody with an active ester of the macrocyclic chelating agent DOTA, radiolabeled the conjugate with either ^{111}In or ^{90}Y, and studied the antibody distribution and therapy, respectively, in athymic mice bearing xenografts of MCF7/HER2/neu, a human breast cancer cell line transfected with the HER2/neu oncogene. For the biodistribution of ^{111}In-labeled DOTA-4D5, a high specificity of tumor localization (30% ID/g) was seen with a tumor-to-blood ratio of greater than 2 at 48 h postinjection. Compared to a previously published study with ^{125}I-labeled 4D5 in beige nude mice bearing NIH3T3/HER2/neu xenografts [De Santes et al. (1992) *Cancer Res. 52*, 1916−1923], ^{111}In-labeled 4D5 antibody gave superior antibody uptake in tumor (30% ID/g vs 17% ID/g at 48h). In the therapy study, treatment of the nude mice bearing MCF7/HER2/neu xenografts with 100 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5 caused a 3-fold reduction of tumor growth compared to untreated controls (injected with human serum albumin) in 40 days. Treatment of animals with 100 μCi of nonspecific antibody ^{90}Y-labeled DOTA-Leu16 (3 μg) had no tumor growth inhibition. Treatment with unlabeled DOTA-4D5 (3 μg) had a slight effect on tumor growth compared to untreated controls. When analyzed at the level of single animals, no effect was seen in seven of nine animals; however, in two of the animals, tumor growth inhibition was observed. Although a cold antibody therapeutic effect was unexpected at this dose level (3 μg), it may be possible that in some animals that 3 μg of antibody of ^{90}Y-labeled DOTA-4D5 augmented tumor growth reduction. To further explore the effects of cold antibody treatment alone, animals were treated with 100 or 400 μg of unlabeled 4D5 administered in two doses. These animals showed a 1.7−1.8-fold reduction in tumor growth over 28 days, a result less than that obtained with RIT only.

INTRODUCTION

The HER2/neu receptor, the product of the c-erbB2 oncogene, has been the target of monoclonal antibody clinical therapeutic trials (*1*−*3*). The oncogene encodes a 185 000 transmembrane phosphoglycoprotein (*4*−*6*), is amplified in 20−30% of breast carcinoma (*7*−*9*), and is linked to poor prognosis (*10*). Several studies have suggested that reducing expression of HER2/neu gene product may convey a growth disadvantage on the tumor (*11*, *12*). Many preclinical trials have shown that certain anti-HER2/neu antibodies can inhibit the growth of HER2/neu-overexpressing tumors (*12*−*16*). In particular, the anti-HER2/neu monoclonal antibody 4D5, when used with cisplatin, has been shown to promote drug-induced killing in target cells (*17*, *18*). Recently, Herceptin, the humanized version of the murine 4D5 monoclonal antibody (*19*), was approved by the FDA for the treatment of HER2/neu positive tumors. The approach utilizes a combination of Herceptin and chemotherapeutic agents. In a phase II study in which 37 patients received Herceptin and cisplatin, a 24.3% partial response rate was observed (*2*).

In addition to any biological effects resulting from the direct interaction of anti-HER2/neu antibody with the HER2/neu receptor, anti-HER2/neu antibody can also be used to deliver drugs, toxins, or radioisotopes directly to HER2/neu positive malignant cells. In the case of radioimmunotherapy (RIT),[1] the use of β-emitter radiolabeled antibody is appealing because tumor cells inaccessible to antibody may still be killed by radioimmunoconjugates bound to neighboring cells. DeSantes et al. (*20*) demon-

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[1] Abbreviations: DOTA, 1,4,7, 10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; FITC, fluorescein isothiocyanate; HSA, human serum albumin; BSA, bovine serum albumin; RIT, radioimmunotherapy; RTV, relative tumor volume; MTD, maximum tolerated dose; ABC, avidin−biotin complex.

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strated that animals treated with 400–700 μCi of ^{131}I-labeled anti-HER2/neu 4D5 monoclonal antibody showed marked inhibition of tumor growth; however, the choice of ^{131}I-labeled antibody requires a higher activity level due to dehalogenation in normal and malignant tissues.

Radiometals that are conjugated to antibody via chelates may be a better choice as exemplified by RIT studies with radiometal labeled anti-lymphoma (*21*), anti-colon cancer (*22*), and anti-breast cancer (*23*) antibodies. Bifunctional chelates such as DTPA (*24*) or DOTA (*25*, *26*) derivatives have been successfully conjugated to antibodies giving products with high retention of immunoreactivities and radiolabeled to high specific activities with either ^{111}In, a γ-emitter with a half-life of 67.9 h, or ^{90}Y, a pure β-emitter with a 64.0 h half-life. In the case of HER2/neu positive tumors, Horak et al. (*27*) have demonstrated good tumor specificity, pharmacokinetics, and radioimmunotherapy with ^{212}Pb-labeled, DOTA-conjugated AE1 anti-HER2/neu antibody in an ovarian tumor model; however, their reagent did not provide effective therapy for large established tumors. In addition, ^{212}Pb may not be suitable for treatment of bulky solid tumors due to its short half-life of 10.6 h and the low tissue penetration of α-rays. Our recent studies have focused on the therapeutic effect of ^{90}Y-labeled, DOTA-conjugated anti-CEA antibodies (*22*). In this report, we have studied the feasibility of targeting the HER2/neu protein with DOTA-conjugated anti-HER2/neu antibody 4D5. Biodistributions of the ^{111}In-labeled and therapy efficacy with the ^{90}Y-labeled antibodies were determined in a nude mouse model bearing the MCF7/HER2/neu xenograft.

EXPERIMENTAL PROCEDURES

Cell Line. MCF7/HER2/neu is a human breast tumor cell line expressing HER2/neu and was obtained from Dr. Dennis Slamon (UCLA). Cells were maintained in RPMI 1640, supplemented with 10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM L-glutamine. To establish tumor growth in nude mice, subconfluent monolayer cells were trypsinized in EDTA, washed in PBS, and resuspended in RPMI1640.

Antibody. 4D5 is a murine IgG_1 monoclonal directed against the extracellular domains of p185 (HER2/neu) and was a gift from Dr. Paul Carter, Genentech, Inc. The antibody was conjugated with DOTA, using active ester carbodiimide chemistry, with a DOTA-ester-to-antibody ratio of 100:1 (*25*). The antibody was then radiolabeled at 43° C for 1 h with ^{111}In or ^{90}Y, at pH 5. The labeling efficiency was greater than 80% for both radionuclides. The number of chelates per antibody was 5.0 as determined by a modification of a ^{57}Co(II) binding assay (*28*), except $^{111}InCl_3$ was used. The binding affinity of [^{111}In]-DOTA 4D5 to MCF7/HER2/neu cells was 1.25×10^8 M^{-1}, as determined by Scatchard analysis (data not shown). The immunoreactivity of [^{111}In]DOTA 4D5 as determined by binding to MCF7/HER2/neu cells was 96% by linear extrapolation to binding at infinite antigen excess (*29*). The anti-CD20 murine antibody Leu16 (Becton Dickinson) was conjugated with DOTA active ester as described above.

Flow Cytometry Analysis. Specific binding of 4D5 to HER2/neu gene product on MCF7/HER2/neu cells was assessed by indirect immunofluorescence. Untransfected MCF7 cells which do not express HER2/neu were used as a negative control. MCF7/HER2/neu was used as a HER2/neu positive cell line, and Leu16 a nonspecific antibody control. One million cells in 0.5 mL of PBS/1%BSA were incubated with 0.1 μg of antibody for 1 h on ice. The mixtures were then washed two times, incubated with 1 μg of FITC-conjugated goat anti mouse IgG Fab'_2 fragment (Jackson Immuno Research Labs) for 40 min, washed once with PBS/1% BSA, and then analyzed on a MoFlo flow cytometer (Cytomation, Fort Collins, CO).

Immunohistochemistry. Fresh tumor tissues were excised from the untreated and from the 100 μCi-4D5 treated group 40 days after therapy. Tissues were embedded in O. C. T. (Miles Inc.) and were quick frozen in isopentane. Tissue sections were cut in 4 um thickness and were mounted on Probe-on slides (Vantana Medical System Inc.). Frozen section slides were fixed in cold acetone for 10 min, then washed with PBS three times, 5 min each. All slides were loaded into a Techmate slide holder and stained using the Biotech Techmate 1000 Immunostainer. Sections were incubated 25 min with 5 μg/mL of 4D5, or with 5 μg/mL of Leu16 as a negative control. The antibody–antigen complex was detected by a modified ABC method (30) (Vantana Medical System Inc.) per manufacturer's direction with the Chromagen 3′3-diaminobenzidine.

Animal Model. Three days prior to injection of tumor cells, 2 month old female mice (NCI) weighing 13-21 g were implanted s.c. with 1.7 mg, 60 day release 17 β-estradiol pellets (Innovative Research of America) on the shoulder pads to promote tumor growth. The mice were inoculated s.c. in the flanks with 10 million MCF7/HER2/neu cells resuspended in 0.15 mL of RPMI1640. Solid tumors measuring approximately 100 mm^3 were visible at 12 days postinoculation. The mice were then randomized according to tumor size to prevent any bias in the biodistribution and radioimmunotherapy studies.

Antibody Biodistribution. Groups of five mice per time point bearing MCF7/HER2/neu xenografts were injected with 3.6 μCi of ^{111}In-labeled DOTA-4D5 via tail vein. Animals were sacrificed at 0, 5, 24, 48, 72, and 96 h postinjection. The average tumor weights were 0.13, 0.25, 0.22, 0.29, 0.35, and 0.41 g, respectively. Tumors were dissected and the major organs and blood weighed and the activity measured. The activity expressed in percentage injected dose per gram of tissue was then calculated. The mean values were used to construct biodistribution curves for tumors and normal organs.

Radioimmunotherapy. DOTA-4D5 and DOTA-Leu 16 were labeled with ^{90}Y to specific activities of 33.3 μCi/μg. Groups of nine tumor-bearing mice (average volume 100 mm^3) were injected via tail vein with 50 μCi or 100 μCi DOTA-4D5, 100 μCi DOTA-Leu16, DOTA-4D5, or 1% HSA/saline. Each mouse received equal amounts of protein (3 μg). Tumors were measured twice a week (after antibody injection) for 37 days. Tumor volume was calculated as Length × $Width^2/2$, where length was the longer of the two measurements. The relative tumor volume was calculated as the ratio of tumor volume on that day to its value at the start of therapy. The growth curve was plotted as the average of the relative tumor volume within a group vs time.

To assess the effect of unlabeled 4D5 on tumor growth, groups of eight tumor-bearing mice (average tumor volume 200 mm^3) were also given 100 μg or 400 μg of 4D5 in two doses by i.p. injection on day zero and on day eight. Leu16 (400 μg) in two doses was used as a negative control. Tumor volumes were measured for 28 days, and the growth curves were constructed as above.

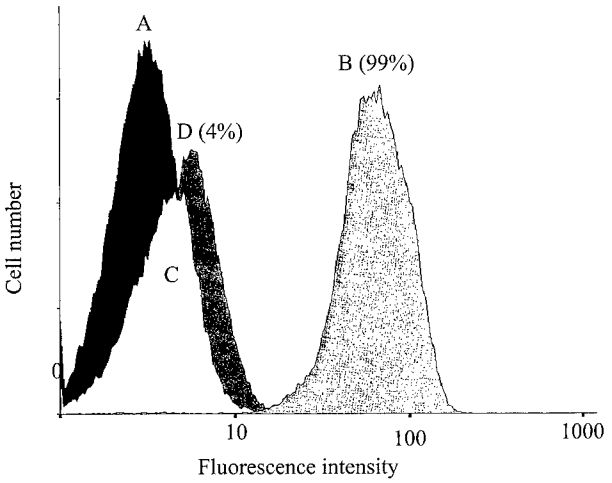

Figure 1. Fluorescent-activated cell sorter analysis of binding of 4D5 to MCF7/HER2/neu and to MCF7 cells. (A) MCF7/HER2/neu control, stained with Leu16, (B) MCF7/HER2/neu stained with 4D5, (C) MCF7 control stained with no primary antibody, (D) MCF7 stained with 4D5. The percent positive cells (vs control) are shown for B and D.

RESULTS

Antibody and Cell Analysis. Indirect immunofluorescence with flow cytometry demonstrated intense, specific binding of the DOTA-4D5 antibody to MCF7/HER2/neu cells (99% positive vs control), but low binding to the parent MCF7cell line (4% vs control), a cell line with limited expression of the HER-2/neu gene product (Figure 1). The 4D5 antibody was conjugated to DOTA, a macrocyclic chelator, using an active ester chemical method (*25*). The DOTA-4D5 conjugate was shown to have a DOTA/antibody ratio of 5/1, radiolabeled with either ^{111}In or ^{90}Y to high efficiency (>90%), and retained high immunoreactivity (>90%) when tested in a cell-binding assay. Nude mice bearing MCF7/HER2/neu xenografts were used as a model for tumor targeting with radiolabeled DOTA-4D5. Immunohistochemical analysis of HER2/neu expression in both treated and nontreated tumors showed membrane staining in approximately 99% of the tumor cells, while staining with the control Leu16 antibody showed only background staining (Figure 2). These results demonstrate that the HER2/neu xenografts continue to produce HER2/neu on the cell surface while growing in nude mice, including after treatment with [^{90}Y]DOTA-4D5.

Antibody Biodistribution. The biodistribution of [^{111}In]DOTA-4D5 was evaluated in nude mice bearing MCF7/HER2/neu xenografts. Groups of five mice per time point were injected with 3.6 μCi of [^{111}In]DOTA-4D5 via the tail vein. Biodistribution studies performed with [^{111}In]DOTA-4D5 showed progressive accumulation of the antibody in MCF7/HER2/neu tumors ranging from 8% ID/g at 5 h to 24% ID/g at 96 h postinjection, as shown in Figure 3 and Table 1 of the Supporting Information. At the same time points, the activity present in blood decreased from 23% to 10% ID/g. The tumor-to-blood ratio increased from 0.36 at 5 h to 2.4 at 96 h postinjection. Organs with significant nonspecific accumulations of antibody included the liver, kidneys, and lung, which ranged from 6 to 11% ID/g at 5 h; however, the accumulations decreased to less than 5% ID/g at 96 h. The results demonstrated specific accumulation of antibody in tumors compared to other major organs.

Radioimmunotherapy. To assess the therapeutic efficacy of DOTA-4D5 in athymic mice bearing MCF7/HER2/neu xenografts, we injected 50 or 100 μCi of ^{90}Y-labeled DOTA-4D5 (3 μg) into groups of nine mice. Control groups were injected with 100 μCi of ^{90}Y-labeled DOTA-Leu16 (3 μg), unlabeled DOTA-4D5 (3 μg), or 1% human serum albumin in saline. With the exception of the HSA control group, all groups received a single injection of 3 μg of antibody. Tumor volumes were measured twice weekly. The average relative tumor volume (RTV) was calculated as the ratio of tumor volume on that day to the volume at the start of therapy. The average relative tumor volume within each group was compared over 37 days (Figure 4). Mice injected with 50 μCi ^{90}Y-labeled DOTA-4D5 showed a 2-fold reduction in tumor growth at day 37, as compared to mice injected with serum albumin/saline [RTV 4.69 vs 9.30, $p < 0.04$, t-test; Table 2 (Supporting Information)]. When the

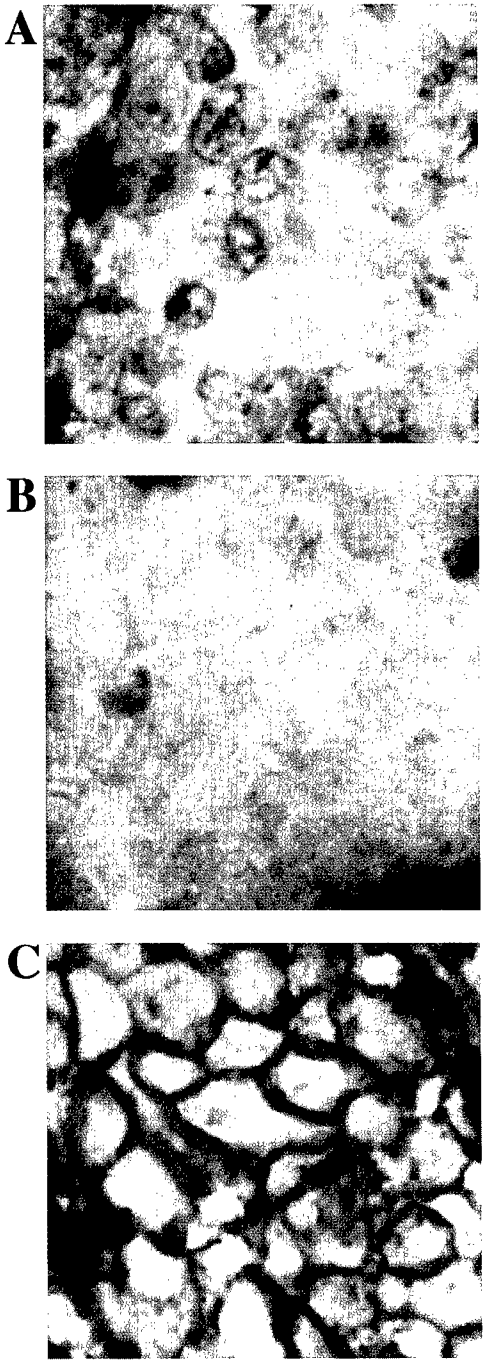

Figure 2. Immunohistochemical frozen sections of MCF7/HER2/neu tumor excised from untreated mice 40 days after therapy. Tumor frozen sections probed with (A) no primary antibody, (B) Leu16 (control), and (C) 4D5.

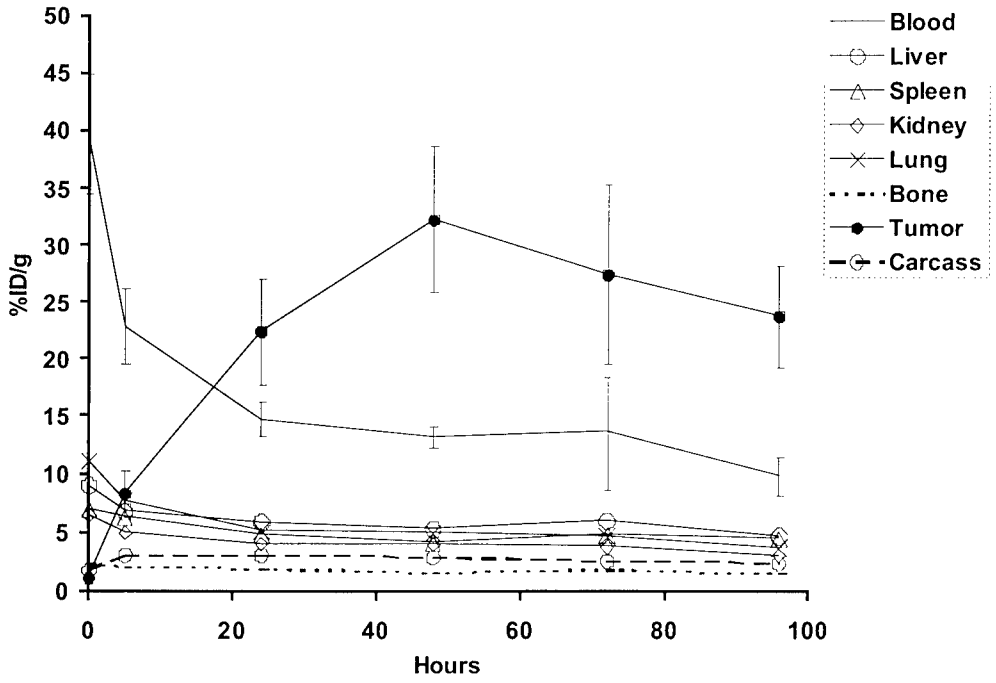

Figure 3. Biodistribution of ^{111}In-labeled DOTA-4D5 in nude mice bearing MCF7/HER2/neu xenografts. Groups of five mice were injected with 3.6 μCi (2.4 μg) of ^{111}In-labeled DOTA-4D5. At 0, 5, 24, 48, 72, and 96 h postinjection the tumors and normal organs were excised, weighed and the ^{111}In activity measured. The mean %ID/g (±std dev) at each time point are shown for blood, liver, spleen, kidney, lung, bone, tumor, and carcass.

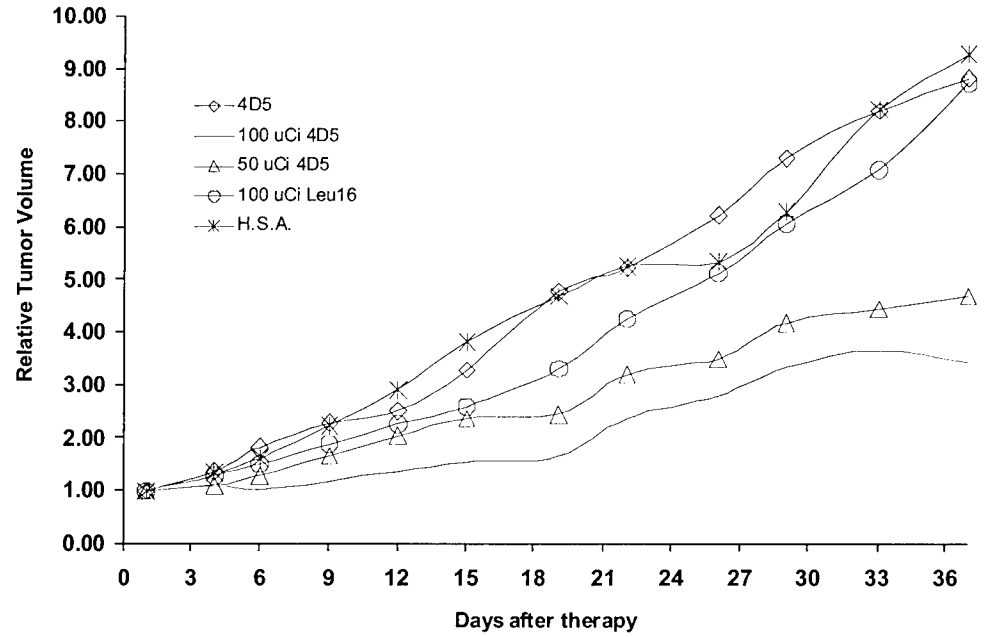

Figure 4. Radioimmunotherapy of established MCF7/HER2/neu tumors in nude mice with ^{90}Y-labeled DOTA-4D5. Groups of nine mice were injected i.v. with (◇) 3 μg of unlabeled DOTA-4D5, (—) 100 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5, (△) 50 μCi (3 μg) of ^{90}Y-labeled DOTA-4D5, (○) 100 μCi (3 μg) of ^{90}Y-labeled DOTA-Leu16, or (*) 1% HSA/saline. The tumor volume for each mouse at the time indicated was normalized to the tumor volume at the start of treatment. Mean values (±std dev) for all animals in each group are shown in Table 2 (Supporting Information).

amount of activity was increased to 100 μCi, the retardation of tumor growth reached 2.7-fold (RTV 3.42 vs 9.30, $p < 0.02$, t-test). Treatment with ^{90}Y-labeled control antibody, DOTA-Leu16, however, did not slow tumor growth over the course of study (RTV 8.77 vs 9.30). Similarly, treatment with unlabeled DOTA-4D5 did not result in significant tumor growth retardation compared to the RIT-treated animals (RTV 8.83 vs 9.30). However, if the data is analyzed at the level of single animals, we noted that two of nine animals treated with unlabeled DOTA-4D5 showed tumor growth inhibition (Table 2, Supporting Information). If a single animal had shown this result, we might have dismissed it; however, the finding occurred in two animals and the effect was not seen in the serum/albumin/saline-treated controls.

To assess the effect of cold antibody alone on tumor growth, we treated mice with higher levels of unlabeled 4D5 (Figure 5). Mice injected with 100 μg of 4D5 showed a 1.65-fold reduction in tumor growth at day 28, as compared to mice injected with 400 μg of Leu16 (RTV 4.09 vs 6.75, $p = 0.15$, t-test; Table 1). Mice injected with 400 μg of 4D5 showed a 1.80-fold reduction (RTV 3.74 vs

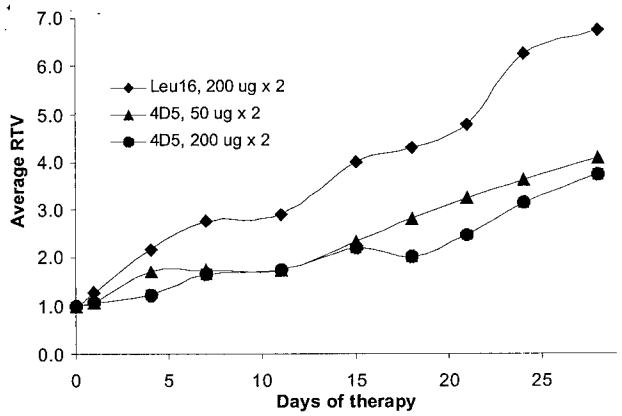

Figure 5. Immunotherapy of established MCF7/HER2/neu tumors in nude mice with 4D5. Groups of eight mice were injected i.p. on day zero and on day eight with (▲) 50 μg of 4D5, (●) 200 μg of 4D5, or (◆) 200 μg of Leu16. The tumor volume for each mouse at the time indicated was normalized to the tumor volume at the start of treatment. Mean values (±std dev) for all animals in each group are shown in Table 1.

6.75, $p = 0.11$, t-test). On the basis of these observations, we concluded that even at the level of 3 μg of antibody treatment, the majority of the RIT effect is due to radiation but not to the cytotoxic effects of antibody alone. Thus, despite the unexpected effect of cold antibody, our conclusions remain the same, i.e., that RIT treatment with the HER2/neu specific antibody has a statistically significant effect on tumor growth compared to the unlabeled antibody-treated group. Finally, it should be noted that while [^{90}Y]DOTA-4D5 slowed tumor growth, tumor regrowth occurred after about 19 days after therapy, as shown in Figure 4. Statistical analysis (ANOVA single factor) showed that there was no statistically significant difference among the five groups after 19 days.

DISCUSSION

To use HER2/neu as a target for radioimmunotherapy, it is necessary to show that an anti-HER2/neu antibody can be successfully radiolabeled without loss of immunoreactivity, that the radiolabeled antibody can target to HER2/neu positive tumors with good tumor to normal tissue ratios, and that RIT shows a positive effect compared to appropriate controls in a preclinical model. In this study, we utilized the previously described MCF7/HER2/neu cell line (*31*) as a model system. This cell line is derived from MCF7, a commonly used estrogen-sensitive cell line in tumor models of breast cancer. The cell line grows well in nude mice bearing slow estrogen release implants and, as shown in Figure 1, reacts strongly with the anti-HER2/neu antibody 4D5. In addition, HER2/neu expression was demonstrated in both treated and untreated tumors in the nude mouse xenograft model (Figure 2). We chose the monoclonal antibody 4D5 because its reactivity with HER2/neu is well established (*18*, *20*, *31*–*33*) and its humanized counterpart, Herceptin, is approved for clinical use. Furthermore, we were able to conjugate 4D5 with the macrocylic chelator DOTA and radiolabel it with ^{111}In to high specific activity (>30 μCi/μg, 90% labeling efficiency) while retaining high immunoreactivity (>90%) and good targeting to the MCF7/HER2/neu xenografts in nude mice (Figure 3, Table 1 of the Supporting Information). In comparing our results to those of De Santes et al. (*20*) who used the HER2/neu-transfected cell line NIH3T3/HER2/neu in beige/nude mice and ^{125}I-labeled 4D5, we obtained a higher maximum tumor uptake (30% ID/g vs 17% ID/g) and similar tumor to normal tissue ratios at 48h (e.g., tumor/liver uptake for ^{125}I-labeled 4D5 was 6.3, and for ^{111}In-labeled 4D5, the ratio was 6.1). The better tumor uptake may have been due to either dehalogenation of the ^{125}I-labeled antibody in tumor or to the better expression of HER2/neu in the MCF7/HER2/neu xenograft model. The former effect is more likely and has been shown to be a major advantage of radiometal labeled antibodies over radioiodine labeled antibodies (*34*–*37*). The favorable biodistibutions prompted us to perform RIT in the same model with ^{90}Y-labeled DOTA-4D5.

RIT with ^{90}Y-labeled DOTA-4D5 showed a significant tumor growth inhibitory effect compared to untreated (serum albumin/saline) controls, antibody only controls, or ^{90}Y-labeled DOTA-Leu16 controls (Figure 4 and Table 2 of the Supporting Information). The growth inhibitory effect was seen over a period of 19 days, after which tumor regrowth occurred and there was no further significant difference in growth rates among the four groups. In this model, where a single dose of RIT was given, positive effects of the treatment lasted over 6 half-lives of the isotope used (^{90}Y, 64 h). It is possible that a stronger effect would have been obtained if the animals were retreated or if a larger dose of radioactivity were administered. In this respect, we found a stronger effect for 100 μCi/animal compared to 50 μCi/animal (RTV 3.42 vs 4.69). Since no animals died with the 100 μCi dose of RIT, it may be possible to administer even higher doses. In previous studies using ^{90}Y-labeled anti-CEA antibodies in a colon cancer model with anti-CEA antibodies, we found an MTD of 120–130 μCi, which could be increased to 200 μCi if the animals were supported with bone marrow stem cells (*38*). Since the major toxicity in studies of this kind are hematologic and due to circulating radiolabeled antibody, the calculation of the appropriate highest dose depends on blood levels of the antibody (which differ depending on the tumor antigen and antibody system). In this study, several of the animals in the control (^{90}Y-labeled Leu16) RIT group died, since the circulating levels of ^{90}Y-labeled DOTA-Leu16 were higher than for ^{90}Y-labeled DOTA-4D5. The difference in circulating levels between the two antibodies is due to tumor uptake and possibly antigen–antibody clearance to the liver in the one case, but not in the other. The latter possibility cannot be verified, since soluble HER2/neu levels weren't measured.

In the RIT studies, we included an unlabeled antibody control group in which antibody was administered at the same protein dose (3 μg, 0.1 mg/kg) as in the RIT-treated animals. Since this dose was well below what is considered to be a therapeutic level in other studies with this cell line (*31*), we expected no antitumor effects. However, two of nine animals showed significant tumor growth inhibition. In one animal, the tumor was very small (34 mm^3), while the tumor was larger (75 mm^3) in the second animal. Even with these animals included, there was a significant difference between this control group and the RIT-treated groups until 19 days ($p < 0.02$, ANOVA single factor). If these two animals are excluded from this control group, the difference among the RIT groups is even greater ($p < 0.01$ at 19 days and $p < 0.04$ at 37 days, ANOVA single factor). Since the effect was seen for two animals, we doubt that the effect represents a random biological variance. In fact, in a separate experiment, we found that antibody doses 30 times higher (100 μg) had definite antitumor effects (Figure 5 and Table 1). Furthermore, in a slightly different model system, Baselga et al. (*39*) observed antitumor effects at multiple

Table 1. Average Relative Tumor Volumes vs Days after Therapy[a]

	days of therapy									
treatment	0	1	4	7	11	15	18	21	24	28
4D5,	1.00	1.06	1.71	1.75	1.74	2.32	2.82	3.24	3.62	4.09
50 μg × 2		(0.25)	(0.44)	(0.47)	(0.82)	(1.23)	(1.68)	(2.03)	(2.36)	(2.86)
4D5,	1.00	1.08	1.24	1.65	1.73	2.20	2.02	2.47	3.13	3.74
200 μg × 2		(0.36)	(0.52)	(0.61)	(0.63)	(0.60)	(0.50)	(0.86)	(1.22)	(1.66)
Leu16,	1.00	1.28	2.16	2.76	2.89	4.01	4.29	4.77	6.25	6.75
200 μg ×2		(0.25)	(1.43)	(1.62)	(1.80)	(2.90)	(2.89)	(3.36)	(5.75)	(6.10)

[a] Groups of eight mice bearing MCF7/HER2/neu xenografts were injected on day zero and on day 8 with 50 μg of 4D5, 200 μg of 4D5, or 200 μg of Leu16. Mean value (±std dev) for all animals in each group are shown.

antibody doses in the 0.1 mg/kg range. However, using the same cell line as in our study (MCF7/HER2/neu) and the same antibody (4D5), Pietras et al. (*31*) found that antibody doses 100 times higher were required to produce some antitumor effects. Overall, we believe that a small single dose of antibody was not responsible for the antitumor effect in our model RIT system.

In the RIT studies reported by De Santes and coworkers (*20*), ^{131}I-labeled 4D5 was used to treat NIH 3T3/HER2/neu xenografts in beige/nude mice. Significant tumor growth inhibition was observed out to 28 days with regrowth beginning after day 20. The activity required for maximum effect was 400 μCi, compared to 100 μCi in our study. Significant growth inhibition was also observed for the ^{131}I-labeled irrelevant antibody control, probably due to the high nonspecific doses characteristic of ^{131}I. It is also known that ^{131}I-labeled 4D5 is rapidly internalized in HER2/neu positive cells, followed by dehalogenation and excretion of the radioiodine (*20*). Metabolism of radiometal-labeled antibodies is much slower, allowing accumulation of radiomental within the cell and thus exposure of the cell to higher doses of radiation (*35*, *40*–*41*). Other differences between the two studies can be ascribed to the differences in growth rates of the two cell lines in the xenograft model. In untreated controls, the NIH3T3/HER2/neu tumor reached 10 cm^3 in 15 days compared to only 1.0 cm^3 in 40 days for the MCF7/HER2/neu model. It is likely that the slower growing tumors represent a more realistic model for human breast cancer and that their response to therapy is accordingly lower.

In the RIT studies reported by Horak et al. (*27*), ^{212}Pb-labeled DOTA-conjugated anti-HER2/neu antibody AE1 was used to treat nude mice bearing HER2/neu positive SK-OV3 xenografts. When small tumors (average mean size 15 mm^3) were treated, significant growth inhibition out to 60 days was observed with 10 or 20 μCi of radiolabeled antibody compared to untreated controls or ^{212}Pb-labeled DOTA-anti-TAC (irrelevant antibody control). In their study, tumor regrowth occurred at 60–70 days. When larger tumors were studied (average mean size 146 mm^3), tumor growth inhibition was not observed, suggesting that α-emitters are more effective against smaller tumors, a result in keeping with the short path length of α-emitters (several cell diameters). On the other hand, α-emitters deposit more energy per μCi (6–9 vs 1–2 MeV for ^{90}Y) thus allowing one to use less activity than ^{90}Y for a given experiment. In our study, the mean tumor sizes were approximately 100 mm^3 at the beginning of RIT and a tumor inhibitory effect was observed for ^{90}Y-labeled mAb.

Our preliminary study showed promising results in favor of using radiometal-labeled-anti HER2/neu antibody for treating breast carcinoma. We have shown that the antibody is readily conjugated to DOTA and radiolabeled without loss of immunoreactivity, and that the radiolabeled antibody targets to HER2/neu positive tumors with good tumor to normal tissue ratios, and that RIT shows tumor growth reduction compared to appropriate controls in a preclinical model. However, several issues need to be addressed in order to increase the therapeutic potential. First, although in our therapy groups the tumor growth is significantly reduced, tumor regrowth occurs after 19 days, and few mice were cured. Increasing radiation dose level or giving multiple doses may increase the therapeutic efficacy of the radiometal-labeled antibody. Second, given that even a small amount of cold antibody treatment alone may slow tumor growth, a combination therapy of radiometal-labeled antibody and unlabeled antibody may have additive or synergistic effects on tumor growth reduction. We are currently investigating these possibilities.

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Supporting Information Available: Tables showing biodistribution of ^{111}In-labeled DOTA-4D5 in nude mice bearing MCF7/HER2/neu xenografts and average relative tumor volume vs days after therapy. This material is available free of charge via the Internet at http://pubs.acs.org.

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